A glycosylation mutant of *Trypanosoma brucei* links social motility defects in vitro to impaired colonisation of tsetse in vivo

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ABSTRACT

Transmission of African trypanosomes by tsetse flies requires that the parasites migrate out of the midgut lumen and colonise the ectoperitrophic space. Early procyclic culture forms correspond to trypanosomes in the lumen; on agarose plates they exhibit social motility, migrating *en masse* as radial projections from an inoculation site. We show that an *Rft1-/-* mutant needs to reach a greater threshold number before migration begins, and that it forms fewer projections than its wild-type parent. The mutant is also up to 4 times less efficient at establishing midgut infections. Ectopic expression of *Rft1* rescues social motility defects and restores the ability to colonise the fly. These results are consistent with social motility reflecting movement to the ectoperitrophic space, implicate N-glycans in the signalling cascades for migration in vivo and in vitro, and provide the first evidence that parasite-parasite interactions determine the success of transmission by the insect host.

(148 words)

INTRODUCTION

Tsetse flies (Glossina spp) are the definitive hosts of the unicellular parasite *Trypanosoma brucei,* while a variety of mammals can serve as intermediate hosts. Different sub-species of *T. brucei* cause sleeping sickness in humans and Nagana in domestic animals. The passage of *T. brucei* through the tsetse fly was memorably described as a "journey fraught with hazards" (1) because the majority of parasites are either eradicated or fail to complete the life cycle. When trypanosomes are ingested by a tsetse fly as part of a blood meal bloodstream forms differentiate into early procyclic forms in the midgut lumen. In the first few days of tsetse infection there are two possible outcomes: the parasites are either purged by the fly or they migrate through/around the peritrophic matrix and colonise the ectoperitrophic space. Extraordinarily little is known about this process: teneral (newly hatched) flies are more susceptible to infection, most probably because the peritrophic membrane is not fully formed and it is easier for parasites to gain access to the ectoperitrophic space (2). There is evidence that several hundred parasites from the initial infectious blood meal are founders of the population in the ectoperitrophic space (3). It is not known, however, if these cross the peritrophic matrix individually or if they migrate in groups. The majority of infections in tsetse do not proceed beyond the midgut stage. Completion of the life cycle involves migration of a small number of parasites to the salivary glands, expansion of the founder population as epimastigote forms and the production of metacyclic forms that can be transmitted to a new mammalian host (1, 3-5).

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The different life-cycle stages of *T. brucei* in the fly express characteristic GPI-anchored glycoproteins that are present in several million copies per cell and cover the entire surface. The early procyclic forms, which are detected in the fly midgut for up to 7 days following fly infection (6), are characterised by the presence of the GPI-anchored protein GPEET procyclin and lesser amounts of EP procyclins (7). The late procyclic forms found in the ectoperitrophic space are negative for GPEET, but continue to express EP1 and EP3 procyclin, both of which are glycosylated (7). In addition to these major surface glycoproteins, trypanosomes express other, less abundant membrane proteins, many of which have the capacity to be modified by carbohydrates (8-11).

Early and late procyclic forms are usually cultured in liquid medium, but they can also proliferate on a semi-solid surface (12). When early procyclic forms are pipetted onto an agarose plate, the parasites first replicate at the inoculation site and aggregate in groups. Upon reaching a threshold cell number, they migrate outwards, resulting in the formation of radial projections or spokes (12, 13). This form of coordinated group movement has been termed social motility (SoMo), based on similar behaviour in bacteria (13). Radial projections from two communities growing on the same plate reorient to avoid encountering each other, suggesting that the parasites produce and sense a repellent. Late procyclic forms can also grow to high densities on plates. Although these do not exhibit SoMo, they do produce substances that deflect the path of early procyclic forms (12). It is evident that the coordination of mass movement on plates requires cell-cell signalling, either through secreted factors or direct cell contact.

In this context it has recently been shown that the knockdown of either of two adenylate cyclases at the flagellar tip results in a hypersocial phenotype, the production of more radial projections (14, 15). Somewhat surprisingly, none of the procyclins is required for SoMo (12). The three mutants so far found to be defective are all motility mutants (13, 16).

Rft1 is an endoplasmic reticular protein involved in the conversion of $Man_5GlcNAc_2$-PP-dolichol (M5-DLO) to M9-DLO, the precursor for N-linked glycans (17-19). The protein is essential in yeast; in humans mutations have been linked to congenital disease and glycosylation defects (20). Recently, an *Rft1* knockout was generated in procyclic forms of *T. brucei* (19). The null mutant accumulated M5-MLO, but had normal levels of mature dolichol-linked oligosaccharide and was capable of glycosylating proteins. It also had a slightly longer population doubling time than its parent (~15 h versus ~12 h in liquid culture) and binding of the lectin concanavalin A (Con A) was reduced by 75%, but no other defects were apparent. An addback expressing an ectopic copy of *Rft1* showed wild-type levels of Con A-binding, confirming that the phenotype was linked to the presence or absence of the gene (19). N-linked glycosylation is known to play a pivotal role in in the folding, quality control, stability and function of surface and secreted proteins (21, 22). It can also be a determinant of signal transduction and host-pathogen interactions (23) and has been implicated in density sensing and adhesion during the development and swarming behaviour of *Dictyostelium* (24-26).

It has been a matter of some debate whether SoMo is a phenomenon that only occurs in culture or if it is a manifestation of an event that occurs in vivo (27). Based on its restriction to early procyclic forms, it has been hypothesised that SoMo reflects the migration from the midgut lumen to the ectoperitrophic space (12). We show that the Rft1 null mutant is compromised both in its ability to perform SoMo and in the establishment of midgut infections. This provides the first evidence that SoMo reflects a specific event in vivo and highlights the importance of parasite-parasite interactions in allowing them to colonise their host.

MATERIALS AND METHODS

Trypanosomes. The parental strain *T. b. brucei* Lister 427, *Rft1 -/-* null mutant and addback were described previously (19). Early procyclic forms were cultured in SDM-79 containing 10% foetal bovine serum and 20 mM glycerol or on plates containing the same medium supplemented with 0.4% agarose (12).

Flow cytometry. This was performed as described (28). Briefly, cells were fixed with 4% paraformaldehyde and 0.2% glutaraldehyde for 20 minutes at room temperature and were then blocked for 1 h with 4% bovine serum albumin. Immunostaining with anti-EP and GPEET antibodies was performed as described by Vassella et al. (6). The secondary antibodies Alexa-Fluor 488 goat anti-rabbit and Cy3 goat anti-mouse (Invitrogen) were used at dilutions of 1:1000. Ten

thousand cells per sample were analysed using a FACscalibur (BD Biosciences) and analysed with FlowJo.

Plating and social motility assay. Plates were poured as described and used within 24 h (12). Following inoculation, the plates were sealed with parafilm and incubated at 27° with 5% CO_2. To determine the cell number at the point when migration started, cells were spotted onto the surface of an agarose plate and incubated as described above. The inocula for the wild type and addback were $2x10^5$ cells and the inoculum for the Rft1 null mutant was $4x10^5$. Plates were inspected every 8 - 12 hours. At the point when radial projections became visible trypanosomes were washed from the plate in 1 ml of serum-supplemented SDM-79 and counted using a haemocytometer. Only viable cells were scored. With these inocula the majority of cells remained viable. Plates were photographed as described (12).

Fly infections. *Glossina morsitans morstinans pupae* were obtained from the Department of Entomology, Slovak Academy of Science, Bratislava. Infection of teneral flies and grading of infections were performed as described (29). The parental line was tested 3 times, and the knockout and addback twice, in independent experiments.

Imaging of trypanosomes in liquid culture. Logarithmically growing cultures were diluted to 4 x 10^6 cells ml^{-1} in complete medium and 10µl were transferred

to a Neubauer haemocytometer counting chamber. Images were taken every 0.5s for 20 seconds with a Leica DFC360FX monochrome CCD (charge-coupled-device) camera mounted on a Leica DM5500 B microscope with a 20x objective using LAS AF software (Leica). Movies were generated with ImageJ.

RESULTS AND DISCUSSION

We first compared the ability of the *Rft1* null mutant (19) and its wild-type parent to perform SoMo on agarose plates. Before embarking on these experiments we determined the number of early (GPEET-positive) procyclic forms in each population since only early procyclic forms show SoMo (12). The parent, knockout and addback were 94.4, 87.6 and 91.9% GPEET-positive, respectively, and expressed similar levels of GPEET and EP procyclins (Figure 1A), indicating that underglycosylation did not cause obvious changes in surface architecture. Initially we had difficulty in culturing the null mutant on agarose plates as the cells tended to die. If a larger inoculum was used the mutant survived and replicated, but in contrast to the parental control, colonies did not produce spokes over a 5-day period (Figure 1B). If plates with the null mutant were incubated for longer, radial projections eventually formed, but the numbers were consistently lower than for the parental line (Figure 2A and B). The addback derived from the knockout (19) did not exhibit difficulties in growing on plates, and formed similar numbers of projections as the wild type and at a similar rate (Figure 2A and B). This confirmed that the phenotypic differences were due to

Rft1. Bearing in mind that the mutant replicated more slowly than its parent, and that the cells needed to reach a threshold density on plates, a trivial explanation would be that the knockout took longer to generate this number of cells. We therefore performed a series of experiments in which the number of viable cells was determined at the point when colonies began to form projections (Figure 2C and D). The threshold numbers for wild-type *T. brucei* (1.85 ±0.47 x10^6) and the addback (1.64 ±0.17 x10^6) were in excellent agreement with the number previously determined for strain AnTat 1.1 (12). In contrast, the threshold for the knockout was 3.86 ±0.83 x10^6. Taken together with the lower number of radial spokes, this implies that cells lacking Rft1 either produce lower amounts of the factor stimulating migration (or a less active form of it), or are not as receptive to the signal. As mentioned above, trypanosomes that show motility defects in liquid culture are compromised in their ability to migrate on plates (13, 16). The motility of the Rft1 null mutant was normal, however (Supplemental movies 1-3). In addition, it could still produce and sense the repellent(s) produced by other communities, resulting in projections reorienting and avoiding each other (Figure 2B). In this respect there was no indication that it differed from the wild type.

We next investigated whether a lack of Rft1 affected the ability of the knockout to establish midgut infections. To date, a knockout of GPI8, the transamidase that transfers the preformed GPI anchor to protein precursors, is the only mutant to show pronounced defects in infecting the midgut (30, 31). Teneral flies were infected with the parental line or one of the mutants and monitored for the prevalence and intensity of midgut infections. Infections were

graded into 4 categories (negative, weak, intermediate and heavy) as described previously (29). A first experiment was performed with the wild type and the null mutant; a second experiment also included the addback mutant. Flies were dissected 3 and 14 days post infection (dpi; Figure 4). Dissections at 3 dpi determined whether trypanosomes could survive in the fly at all, while those at 14 dpi determined whether they had succeeded in establishing an infection. In both experiments the wild-type parental line and the knockout gave very similar profiles at 3 dpi, indicating that *Rft1* was not crucial for survival of early procyclic forms in the midgut lumen. At 14 dpi the knockout showed a 2-4 fold lower prevalence of established infections than the wild type. When infections did occur, however, their intensities were similar (mostly heavy infections). This indicates that if the null mutants manage to reach the ectoperitrophic space they can proliferate normally. Once again, expression of the ectopic copy in the addback rescued the phenotype, confirming that *Rft1* influences the ability of trypanosomes to colonise the midgut and/or resist clearance by the fly.

In conclusion, we provide the first evidence for a link between the ability of parasites to perform SoMo and to establish midgut infections, consistent with SoMo reflecting the migration from the lumen to the ectoperitrophic space. Furthermore, these results implicate N-linked glycans in the biogenesis, stability or activity of the migration factor(s) and/or components of the signalling cascade for SoMo, and the function of factors promoting colonisation in vivo. The N-glycans on the EP procyclins and the transmembrane protein PSSA-2 can be excluded, as these proteins are neither essential for SoMo (12) nor for

establishing midgut infections (6, 9). However, both adenylate cyclases that regulate SoMo are glycosylated (14). Moreover, there are a number of surface-associated enzymes (10, 11), nutrient transporters (8) and flagellar components (32, 33) that are (potentially) N-glycosylated and might conceivably play a role in SoMo, as well as additional adenylate cyclases that are differentially expressed between early and late procyclic forms (12).

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FIGURE LEGENDS

Figure 1

Procyclin expression and social motility phenotypes.

A. Surface expression of EP and GPEET procyclin on wild-type 427, *Rft1* knockout (KO) and addback cells. Trypanosomes were co-stained with antibodies against EP and GPEET and analysed by flow cytometry. The upper left panel is a negative control in which the primary antibodies were omitted.

B. *Rft1* knockout (left) and wild-type trypanosomes (right) were inoculated onto the surface of a 0.4% agarose plate. The plate was photographed after 5 days incubation at 27°.

WT: wild type; KO: Rft1 knockout.

Figure 2

The *Rft1* knockout (KO) forms fewer projections and requires a higher threshold density than wild type (WT) and addback trypanosomes.

A. Numbers of projections formed by individual communities on agarose plates.

B. Representative examples of plates, showing the number of projections and that the knockout is capable of producing and responding to repellents.

C. Cell number at the point that projections start to form.

D. Representative plates for the data depicted in C.

Figure 3

The *Rft1* knockout produces a lower number of midgut infections. Flies were dissected 3 and 14 days post infection (dpi) and graded for intensity of infections. A. Experiment 1. B. Experiment 2. In both cases the difference between the wild type and knockout at 14 dpi were statistically significant (one tailed Fisher's exact test).

WT: wild type; KO: *Rft1* knockout.

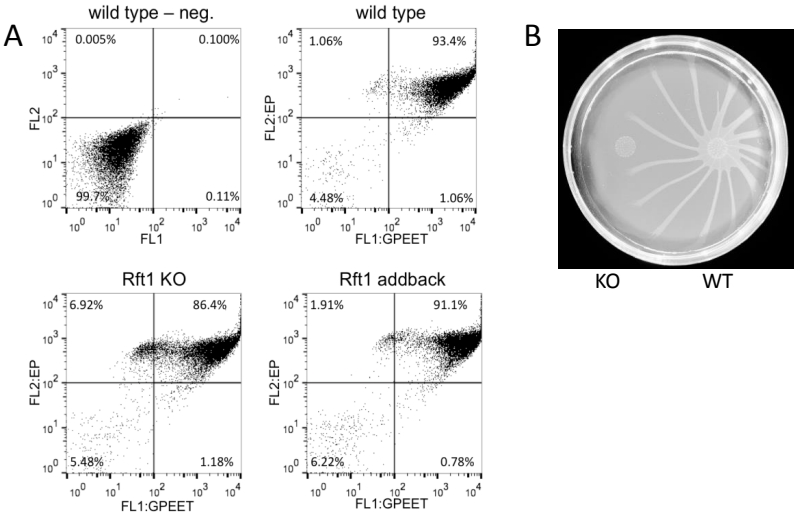

Figure 1 Imhof et al.

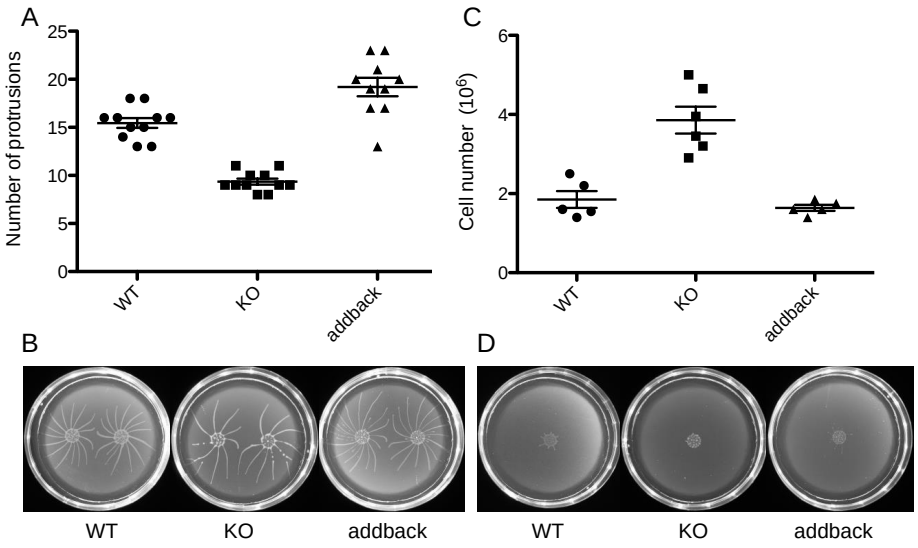

Figure 2 Imhof et al.

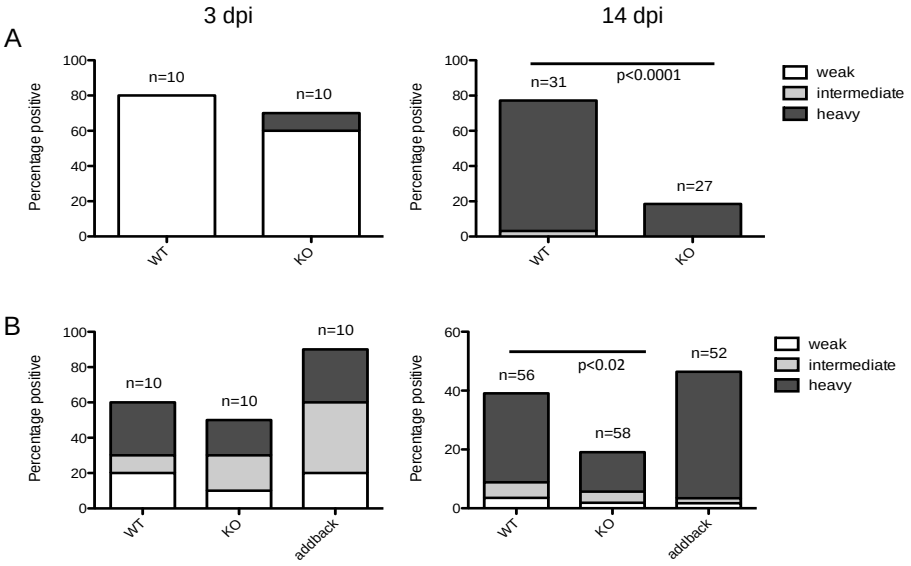

Figure 3 Imhof et al.